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Sucrose Transport into Plasma Membrane Vesicles from Tobacco Leaves by H^+ Symport or Counter Exchange Does not Display a Linear Component

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Abstract. Uptake of [^{14}C]sucrose by plasma membrane vesicles from leaves of tobacco (*Nicotiana tabacum* L.) was measured after the imposition of an inwardly directed proton gradient ($\Delta pH = 2$) and an electrical gradient ($\Delta\psi = -68$ mV, inside negative) across the vesicle membrane. The vesicles were isolated from a microsomal fraction by two-phase partitioning using media that contained 330 mM of either sorbitol or sucrose. Sucrose transport into vesicles isolated using the sorbitol-containing media showed the hallmarks of electrogenic H^+ -symport, as it was highly dependent on ΔpH, could be increased three- to four-fold by $\Delta\psi$, and was abolished by carbonylcyanide *m*-chlorophenylhydrazone (CCCP). Transport of [^{14}C]sucrose into vesicles that were isolated using the sucrose-containing media apparently occurred by counter exchange. Its initial influx also depended on a low external pH, but it was insensitive to CCCP and hardly stimulated by $\Delta\psi$. Both symport and counter exchange obeyed simple Michaelis-Menten kinetics. Transport that depends linearly on the external sucrose concentration could not be detected, indicating that the 'linear' component that has been observed in sucrose uptake by leaf tissues does not represent a transport route that is provided by the sucrose symporter. The potential role of H^+/sucrose-symporters in phloem unloading is briefly discussed.

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Abbreviations: CCCP: carbonylcyanide *m*-chlorophenylhydrazone; sorb-vesicles: vesicles isolated using media that contained 330 mM sorbitol; suc-vesicles vesicles: isolated using media that contained 330 mM sucrose; SUT, H^+ sucrose symporter

Key words: Counter exchange — H^+/sucrose symport — Leaf — *Nicotiana tabacum* L. — Phloem loading and unloading — Plasma membrane vesicles

Introduction

Sucrose is the main product of the photosynthesizing leaf ('source' leaf) from which it is transported through the sieve elements in the phloem of the vascular bundles to 'sink' organs such as roots, developing leaves, seeds and tubers. Sucrose is the major organic solute in phloem sap (Zimmermann & Ziegler, 1975) and its concentration in the sap is usually much higher than that in the mesophyll. This points to a symplasmic discontinuity between mesophyll and the conducting cells of the phloem, implying that photosynthetically produced sucrose is released into the apoplasm and then accumulated in the sieve element/companion cell complex of the minor veins in the leaf. Experiments by Geiger and co-workers constituted a firm foundation for this concept of apoplasmic phloem loading of sucrose and led to the proposition that the process relies on an H^+/sucrose symporter in the plasma membrane of the sieve elements (Geiger, 1975; Giaquinta, 1977, 1983). Strong evidence for H^+/sucrose symporters in leaves has been provided by studies showing that uptake of sucrose in plasma membrane vesicles can be highly stimulated by an artificially created proton motive force across the vesicle membrane (Buckhout, 1989; Bush, 1989; Lemoine & Delrot, 1989).

The first cDNAs encoding plant H^+/sucrose symporters (SUTs) were cloned from spinach (*SoSUT1*) and potato (*StSUT1*) by Riesmeier et al. (1992; 1993). To date, at least 48 genes coding for (putative) sucrose transporters have been identified in 24 plant species (*for reviews*: Williams, Lemoine & Sauer, 2000; Lalonde et al., 2003; Kühn, 2003). Based

Table 1. Kinetics of the sucrose uptake by leaf tissues

Plant species	V_{max}	K_m	V_{max}/K_m	k	References
Broad bean (*Vicia faba* L.)	38 ± 9[a]	3.5 ± 1.1[a]	10.8	2.3 ± 0.2[a]	Delrot & Bonnemain, 1981
Sugar beet (*Beta vulgaris* L.)	64 ± 21[b]	~8[c]	~8	3.5 ± 0.3[b]	Maynard & Lucas, 1982a,b
Tobacco (*Nicotiana tabacum* L.)	57 ± 17[d]	3.3 ± 2.2[d]	17.3	5.0 ± 0.4[d]	our unpublished results

All values are from uptake experiments at pH 5.0. V_{max} in nmol cm^{-2} leaf area h^{-1}; K_m in mM; V_{max}/K_m and k in nmol cm^{-2} leaf area h^{-1} mM^{-1}.

[a] Parameter values ± SE (fitting-derived). Data of Fig. 1 in Delrot & Bonnemain (1981) were originally interpreted to represent uptake by a high-affinity system (K_m = 2.66 mM), a low affinity system (K_m = 35.3 mM), and a linear component (k = 1.21 nmol cm^{-2} leaf area h^{-1} mM^{-1}). The fitting of various rate equations (Borstlap, 1983) revealed, however, that the data could be satisfactorily described by the equation $v = V_{max}[S]/(K_m + [S]) + k[S]$.

[b] Mean values ± SE from eight independent experiments (Fig. 3 in Maynard & Lucas, 1982a and Fig. 3 to 9 in Maynard & Lucas, 1982b).

[c] From Fig. 6 in Maynard & Lucas, 1982a.

[d] Parameter values ± SE (fitting-derived). Uptake was measured as described (Borstlap & Schuurmans, 1988) at nine sucrose concentrations in the range 70 nM to 50 mM.

on sequence similarity the SUTs from dicot species have been classified into three groups. The H^+/sucrose symporters responsible for phloem loading, which have a relatively high affinity ($K_m \approx 1$ mM), belong to the SUT1-subfamily. Some SUT1-members are expressed in epidermal cells of cotyledons and serve the acquisition of sucrose during seed development (Weber et al., 1997; Tegeder et al., 1999) or seed germination (Bick et al., 1998). The SUT2-subfamily includes LeSUT2 from tomato and AtSUC3 (=AtSUT2) from *Arabidopsis*, which possibly function as sugar sensors (Barker et al., 2000; Meyer et al., 2000), PmSUC3 from *Plantago major*, which transports sucrose with a K_m of ~5 mM (Barth, Meyer & Sauer, 2003; Eckardt, 2003), and a ripening-associated SUT (VvSUC12) from grape berries (Davies et al., 1999). The SUT4-subfamily comprises AtSUT4 from *Arabidopsis* and LeSUT4 from tomato (Weise et al., 2000), which are low affinity transporters ($K_m \approx 10$ mM), another ripening-associated SUT (VvSUC11) from grape berries (Davies, Wolf & Robinson, 1999) and one that is expressed in root nodules of *Lotus japonicus* (Flemetakis et al., 2003). Curiously, in carrot (*Daucus carota*) an SUT4-member (*DcSUT1*) is particularly expressed in the lamina of source leaves, suggesting a role in phloem loading, whereas an SUT1-member (*DcSUT2*) is mainly expressed in sink organs such as the storage root (Shakya & Sturm, 1998). SUTs from rice and other monocots fall either into the SUT2- or SUT4-subfamily and it is not clear which monocot SUTs are involved in phloem loading (Aoki et al., 2003).

In the mature, assimilate-exporting leaf, H^+/sucrose transporters seem to be restricted either to the companion cells (Stadler et al., 1995; Stadler & Sauer, 1996; Barth et al., 2003) or, as in solanaceous species (potato, tomato, tobacco), to the sieve elements. SUT1, SUT2, and SUT4 from potato and tomato have all been localized to sieve elements (Kühn et al., 1997; Barker et al., 2000; Weise et al., 2000), while antisense studies have shown that SUT1 from tobacco (NtSUT1) is required for phloem loading in leaves (Bürkle et al., 1998). NtSUT1 is the orthologue of StSUT1, for which a detailed quantitative kinetic model has been proposed (Boorer et al., 1996).

When [^{14}C]sucrose is supplied exogenously to leaf tissue, by far the greatest part of the label accumulates in the minor veins (Fondy & Geiger, 1977; Delrot, 1981), reflecting the importance of sucrose transport into the sieve element/companion cell complex. Interestingly, the concentration dependence of sucrose uptake by leaf tissues can be described by a saturable and an unsaturable or 'linear' component (Table 1). The saturable component can be attributed to phloem loading by a SUT1-type transporter in the sieve element/companion cell complex, but it is not known what transporter is responsible for the linear component. Both a 'sucrose binding protein' (Overvoorde, Fromme & Grimes, 1996) and an SUT4-type transporter (Weise et al., 2000) have been suggested as possible candidates. In addition it has been proposed that the linear component may result from an SUT1-type transporter working in an uncoupled mode (Boorer et al., 1996). Uncoupled sucrose transport by SUTs is also of interest because these transporters may be involved in the efflux of sucrose from the sieve elements in sink tissues (phloem unloading). Evidence for this has been provided by Kühn et al. (2003), who demonstrated that StSUT1 is present in the sieve elements of developing potato tubers, and that its antisense inhibition resulted in delayed tuber development. However, the mechanism by which sucrose is transported out of the sieve elements has not been established.

A linear component has been also reported for the uptake of sucrose by plasma membrane vesicles from leaves. Although in one study the linear component was reported to form part of the proton-motive-force-dependent sucrose uptake (Lemoine et al., 1996), it generally represents the uptake under non-energized conditions (Buckhout, 1989; Lemoine & Delrot, 1989; Williams, Nelson & Hall, 1990). It

has been suggested to result from diffusion or facilitated uptake (Lemoine & Delrot, 1989), either by a 'permease' in the mesophyll cells or by the H^+/sucrose symporter operating in an uncoupled mode (Bush, 1989), but its relationship to the linear component of sucrose uptake by leaf tissue has not been clarified.

In the present work we studied the uptake of sucrose in plasma membrane vesicles from tobacco leaves after the imposition of an inwardly directed proton motive force. To mimic the situation in the intact leaf more closely we also examined the influx of [^{14}C]sucrose into vesicles in which unlabeled sucrose was included. The main conclusions from this study are: (1) that the H^+/sucrose symporter can act in an exchange mode by which the labeled sucrose supplied to the vesicles is exchanged against the unlabeled sucrose inside the vesicles without the net transport of protons or electrical charge, and (2) that the linear component of sucrose uptake by leaf tissues does not represent a transport route that is provided by the H^+/sucrose symporter.

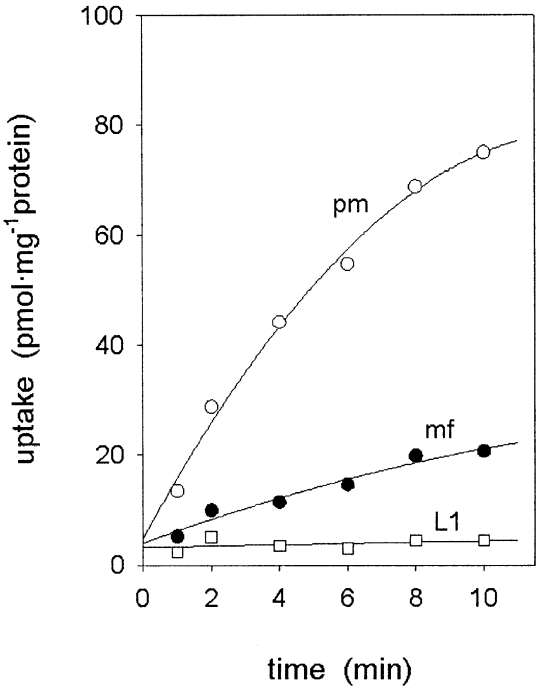

Fig. 1. Uptake of sucrose by vesicles from the microsomal fraction (●), vesicles from the L_1-phase (□), and plasma membrane vesicles (○) after imposition of $\Delta pH + \Delta\psi$. Vesicles were isolated using the sucrose-containing media ('suc-vesicles'), suspended in pH7K-medium, and incubated in pH5Na-medium. Initial influxes (pmol mg^{-1} protein min^{-1}) were: 2.25 ± 0.87 (●); 0.11 ± 0.13 (□); 11.40 ± 1.55 (○).

Materials and Methods

Plants

Tobacco plants (*Nicotiana tabacum* L. cv Xanthi) were grown in pors in a greenhouse. Leaves were collected between 8.00 and 9.00 a.m.

Isolation of Plasma Membrane Vesicles

Plasma membrane vesicles were isolated from a microsomal fraction by aqueous polymer two-phase partitioning (Larsson, Widell & Kjellbom, 1987), as detailed elsewhere (Borstlap & Schuurmans, 2000). For the isolation of 'suc-vesicles' the homogenization medium, the medium in which the microsomal pellet was resuspended, and the two-phase system contained 330 mM sucrose, whereas the washing medium and the pH7 K-medium (in mmole/L: 330 sorbitol, 50 HEPES-KOH, pH 7.0, 39 KCl, 0.1 DTT), in which the vesicles were finally suspended, contained 330 mM sorbitol as an osmoticum. 'Sorb-vesicles' were isolated using media that all contained 330 mM sorbitol as an osmoticum.

Transport Assays

Uptake of [U–^{14}C] sucrose (442 or 475 Ci mol^{-1}) by membrane vesicles was determined using a filtration method (Borstlap & Schuurmans, 2000). Vesicle suspensions were diluted 15-fold in uptake medium that invariably contained sorbitol as an osmoticum. An inwardly directed proton gradient (ΔpH) was imposed across the vesicle membrane by diluting the vesicle suspension in pH5 K-medium (in mmole/L:330 sorbitol, 50 MES-KOH, pH 5.0, 47 KCl, 0.1 DTT). To impose an inside negative membrane potential ($\Delta\psi$) the vesicle suspension was diluted in pH7Na-medium (in mmole/L: 330 sorbitol, 50 HEPES-NaOH, pH 7.0, 39 NaCl, 0.1 DTT). The simultaneous imposition of ΔpH and $\Delta\psi$ was achieved by diluting the vesicle suspension in pH5Na-medium (in mmole/L: 330 sorbitol, 50 MES-NaOH, pH 5.0, 0.1 DTT), whereas pH7 K-medium was used to measure the uptake in the absence of gradients. Unless stated otherwise, the concentration of the labeled sucrose in the uptake medium was 0.76 μM, and valinomycin was supplied at a concentration of 1 μM.

Radiochromatographic Analysis of Vesicle Contents

Plasma membrane vesicles were isolated as described (Borstlap & Schuurmans, 2000) but to avoid interference of the large amounts of osmoticum during the extraction and chromatographic analysis of the vesicle contents, sorbitol was omitted in the washing medium and the pH7 K-medium. Vesicles diluted in the sorbitol-free pH5Na-medium were allowed to take up ^{14}C-sucrose. After 30 min, vesicles from 0.2-ml samples of the incubation mixture were collected on filters (Schleicher & Schuell, ME25, pore size 0.45 μm) and extracted with 80% (v/v) ethanol. The ethanol extract was taken to dryness and the residue was partitioned over a water/chloroform mixture. A sample of the concentrated water layer was spotted on Whatmann MM3. Descending chromatography was run for 30 h in *n*-propanol/benzyl alcohol/formic acid/water (50:72:17:20, by volume). After drying, the paper chromatogram was cut into 0.5 cm-wide strips that were assayed for ^{14}C by scintillation counting.

Results

As compared with the microsomal vesicles, sucrose transport activity in the plasma membrane fraction was enriched ~5-fold (Fig. 1), in fair agreement with the enrichment factors for L-valine transport and the

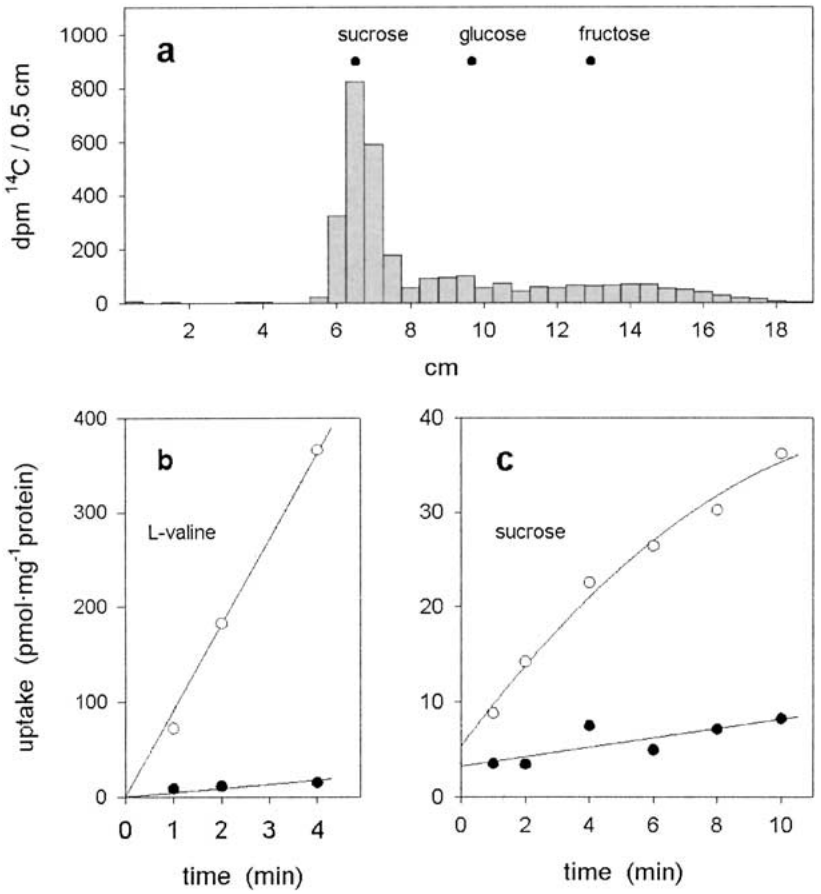

Fig. 2. Radiochromatographic analysis of vesicle contents after the uptake of ^{14}C-labeled sucrose. Plasma membrane vesicles were prepared in media without osmoticum and finally suspended in pH7K-medium without sorbitol (*see* Materials and Methods), (*a*) Radiochromatogram. (*b, c*) Uptake of L-valine (1.27 μM) and sucrose (0.77 μM) was determined in pH5Na-medium without sorbitol (○) or pH7 K-medium without sorbitol (●) in the presence of 1 μM valinomycin.

plasma membrane markers vanadate-sensitive ATPase and glucan synthase II (Borstlap & Schuurmans, 2000).

The chemical identity of the labeled material in vesicles to which ^{14}C-sucrose had been added was assessed by radiochromatography. Virtually all radioactivity extracted from the vesicles co-chromatographed with sucrose (Fig. 2*a*). To avoid disturbing effects of large amounts of osmoticum during extraction and chromatographic analysis, we used for this experiment media without sorbitol (*see* Materials and Methods). The rates of uptake of L-valine and sucrose were comparable to those in vesicles isolated in media with the osmoticum sorbitol (Fig. 2*b,c*; Borstlap & Schuurmans, 2000).

Sucrose Influx into Vesicles Isolated Using the Sorbitol-Containing Media

In vesicles isolated using the sorbitol-containing media ('sorb-vesicles') almost no time-dependent uptake of sucrose could be measured in the absence of gradients. The imposition of ΔpH resulted in an initial influx of sucrose of about 10 pmol mg^{-1} protein min^{-1} . This ΔpH-dependent influx could be stimulated three-fold when Δψ was imposed simultaneously, whereas the imposition of Δψ alone did not result in enhanced uptake (Fig. 3*a*).

The presence of CCCP completely abolished sucrose uptake driven by the artificially imposed proton motive force, whereas the addition of CCCP to vesicles that had accumulated labeled sucrose resulted in a net efflux of the substrate (Fig. 4*a*).

To impose Δψ, suspensions of plasma membrane vesicles in which [K^+] was 50 mM were diluted 15-fold in potassium-free medium. The resulting diffusion potential will depend on the concentration of valinomycin, and maximally amount to −68 mV (Borstlap & Schuurmans, 2000). Addition of valinomycin at a concentration of 1 μM stimulated the sucrose influx about four-fold. Raising the valinomycin concentration to 5 μM did not further stimulate the initial influx, and lowered the maximum uptake (Fig. 5*a*).

Sucrose Influx into Vesicles Isolated Using the Sucrose-Containing Media

Sucrose uptake in vesicles isolated using the sucrose-containing media ('suc-vesicles') also seemed to depend on the imposition of Δ pH, since it was much higher at pH 5 than at pH7 (Fig. 3*b*). But as shown in Fig. 4*b*, addition of CCCP did not affect the initial

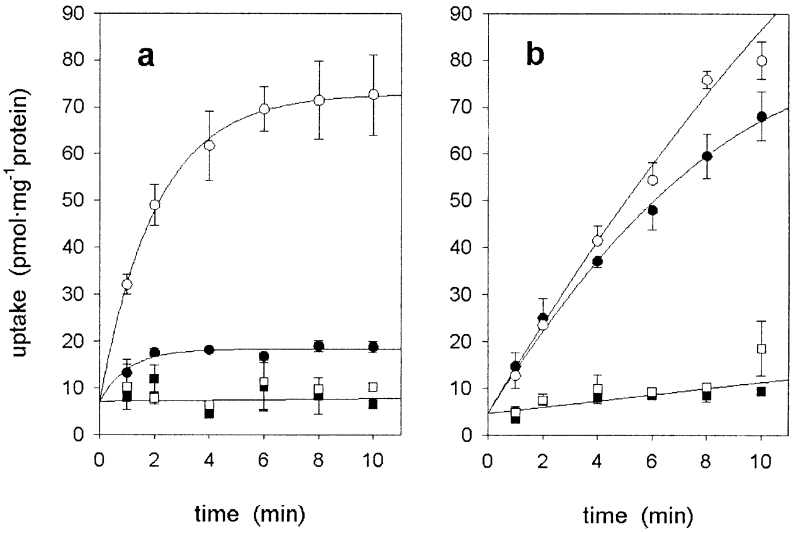

Fig. 3. Sucrose uptake by plasma membrane vesicles after imposition of ΔpH + Δψ (○), ΔpH (●), Δψ (□), or no gradients (■). The incubation media were pH5Na-medium (○), pH5K-medium (●), pH7Na-medium (□), and pH7K-medium (■). The valinomycin concentration in the uptake media was 5 μM. Symbols represent mean values ± SE for two preparations, (*a*) 'Sorb-vesicles' suspended in pH7K-medium. Initial influxes (pmol mg^{-1} protein min^{-1}) were: 10.5 ± 2.6 (●) and 31.8 ± 1.3 (○). Maximum uptakes (pmol mg^{-1} protein) were: 11.2 ± 0.6 (●) and 65.6 ± 0.7 (○). (*b*) 'Suc-vesicles' suspended in pH7K-medium. Initial influxes: 9.85 ± 0.47 (○); 9.41 ± 0.27 (●); 0.66 ± 0.11 (□;■).

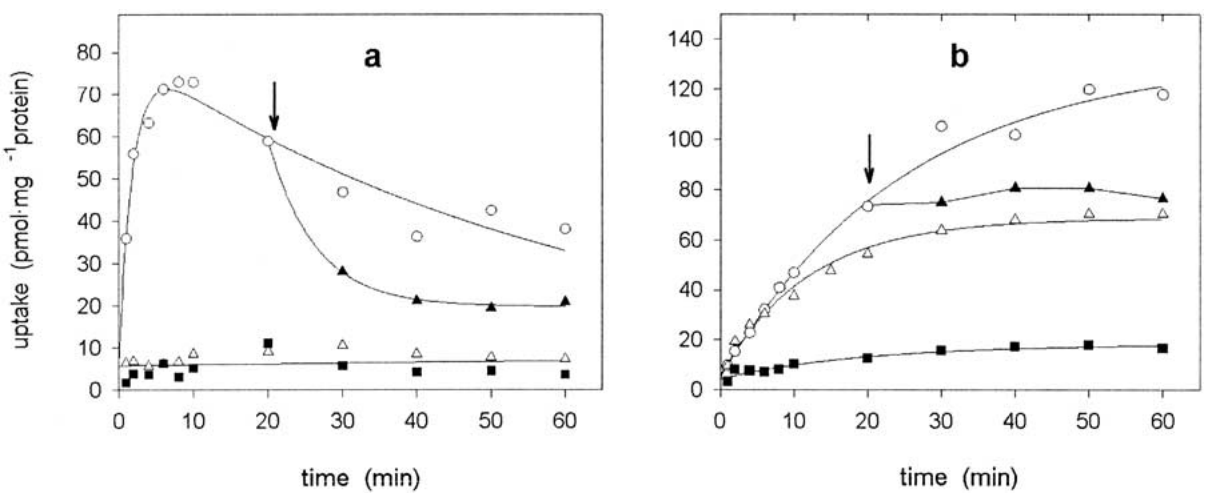

Fig. 4. Effect of CCCP on the uptake of sucrose by plasma membrane vesicles. Vesicle suspensions were diluted in pH7 K-medium (■), pH5Na-medium (○) and pH5Na-medium to which 10 μM of CCCP was added before starting the uptake experiment (Δ) or at the time indicated by the arrow (▲), (*a*) 'Sorb-vesicles' suspended in pH7K-medium. The initial influx of sucrose in pH5Na-medium without CCCP was 40.0 ± 7.0 pmol mg^{-1} protein min^{-1}, and the maximum uptake amounted to 71 ± 3 pmol mg^{-1} protein. (*b*) 'Suc-vesicles' suspended in pH7 K-medium. Symbols represent mean values for two preparations. The initial influxes (pmol mg^{-1} protein min^{-1}) were: 5.34 ± 0.95 (○), 5.13 ± 0.55 (Δ) and 0.71 ± 0.25 (■). Maximum uptake (pmol mg^{-1} protein): 120 ± 4 (○), 71 ± 3 (Δ) and 16 ± 1 (■).

influx of sucrose, indicating that it depended on a low pH in the uptake medium rather than on a transmembrane pH-gradient. However, CCCP prevented further uptake when added to vesicles that had taken up labeled sucrose for 20 min.

The imposition of Δψ alone had no detectable effect, and when imposed together with ΔpH it stimulated the influx of labeled sucrose by no more than 10% (Fig. 3*b*,5*b*).

At a substrate concentration of 0.76 μM, the magnitude of the influx of labeled sucrose ranged from 5 to 10 pmol mg^{-1} protein min^{-1} for the various preparations (Fig. 1, 3*b*,4*b*,5*b*), comparable to or somewhat less than the influx into the vesicles isolated using the sorbitol-containing media after the imposition of ΔpH alone (Fig, 3*a*,5*a*).

Accumulation of [^{14}C] Sucrose in Plasma Membrane Vesicles

The maximum uptake of the *labeled* sucrose that could be attained after the imposition of ΔpH or ΔpH + Δψ was determined in experiments that lasted 1 h. In sorb-vesicles uptake was at a maximum after 5 to 10 min of incubation. Then a net efflux of the labeled sucrose set in, which was more prominent

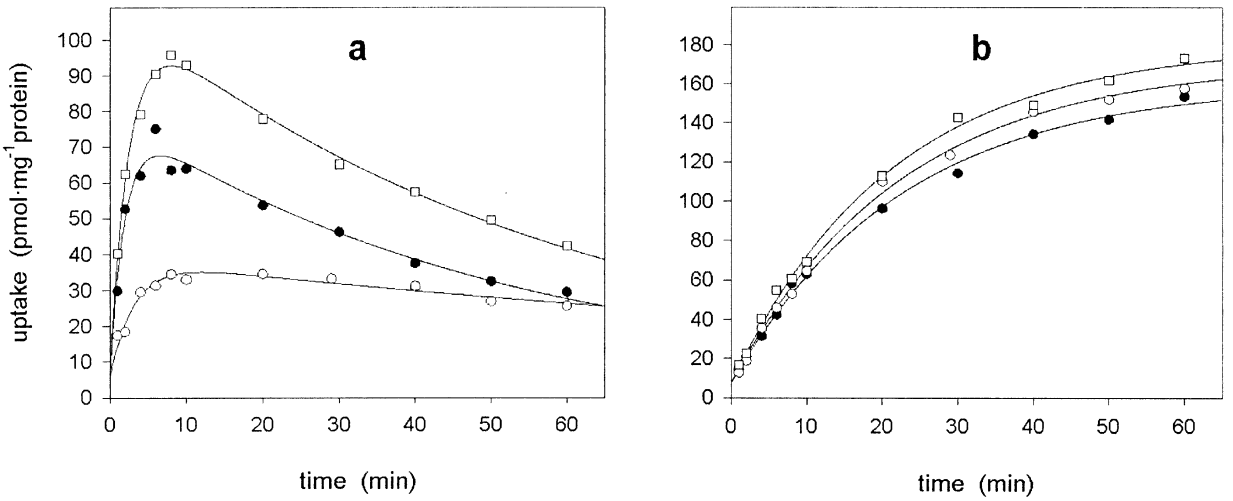

Fig. 5. Effect of the valinomycin concentration on the uptake of sucrose by plasma membrane vesicles. Vesicle suspensions were diluted in pH5Na-medium without valinomycin (◯) or with 1 μM (□) or 5 μM(●) valinomycin. (*a*) 'Sorb-vesicles' suspended in pH7K-medium. Symbols represent the mean values of two preparations. Initial influxes (pmol mg^{-1} protein min^{-1}) were: 10.2 ± 1.7 (◯); 41.0 ± 2.7 (□); 36.2 ± 5.5 (●). Maximum uptakes (pmol mg^{-1} protein): 93 ± 13 (◯); 68 ± 2 (□); 35 ± 1 (●) (*b*) 'Suc-vesicles' suspended in pH7 K-medium. Initial influxes were: 7.31 ± 0.53 (◯); 8.12 ± 0.50 (□); 6.75 ± 0.40 (●). Maximum uptake: 158 ± 3 (◯); 170 ± 3 (□); 150 ± 2 (●).

after the imposition of $\Delta pH + \Delta\psi$ than after the imposition of ΔpH alone. The maximum uptake was 10 to 20 pmol mg^{-1} protein after the imposition of ΔpH (Fig. 3*a*,5*a*), and 60 to 90 pmol mg^{-1} protein after the imposition of $\Delta pH + \Delta\psi$ (Fig. 3*a*,4*a*,5*a*).

The time course of sucrose uptake in suc-vesicles was completely different. Uptake continued to increase during the experimental period of 1 h, approaching a maximum of 120 to 150 pmol mg^{-1} protein (Fig. 4*b*,5*b*), which is nearly two-fold higher than for sorb-vesicles after the imposition of $\Delta pH + \Delta\psi$. The imposition of $\Delta\psi$ in addition to ΔpH slightly increased the accumulation of the labeled sucrose (Fig. 3*b*,5*b*). Even in the presence of CCCP, the labeled sucrose could be accumulated in the vesicles up to ~70 pmol mg^{-1} protein (Fig. 4*b*).

Influx as a Function of the External Substrate Concentration

The concentration dependence of the sucrose influx was assessed in the range 0.76 μM to 10 mM. The results obtained with suc-vesicles are shown in Fig. 6. Analysis of the time-dependent uptake revealed at all concentrations a constant influx during the first three minutes (Fig. 6, *insets at lower left*). The lines representing the time-dependent uptake made distinct intercepts with the y-axis, which were proportional to the external sucrose concentration. On average, the quotient of intercept and external concentration amounted to 6.9 ± 0.5 μmol mg^{-1} protein M^{-1} (Fig. 6, *inset at upper right*).

The concentration dependence of the sucrose influx is shown in Fig. 6 as a plot of $v/[S]_o$ against $^{10}\log[S]_o$. This plot is similar to and has the same advantages as the so called 'displacement curve' (Malo & Berteloot, 1991; Borstlap & Schuurmans, 2000). The curve in Fig. 6 has the form of a simple titration curve, which conforms to Michaelis-Menten kinetics. Curve fitting yielded the following parameter values: V_{max} = 22.0 ± 0.9 nmol mg^{-1} protein min^{-1} and K_m = 1.65 ± 0.08 mM.

Uptake of sucrose in sorb-vesicles was characterized by a lower K_m (Fig. 7; Table 2). After the imposition of ΔpH alone, the K_m was determined at 0.69 ± 0.12 mM. The simultaneous imposition of $\Delta\psi$ lowered the K_m to 0.46 ± 0.02 mM, and increased the V_{max} from 5.46 ± 0.76 to 7.56 ± 0.33 nmol mg^{-1} protein min^{-1}.

Discussion

Plasma membrane vesicles are generally isolated using media that contain a high concentration of osmoticum, e.g. 330 mM of sucrose (Larsson, et al., 1987). Our results show that the presence of osmoticum is not essential for the activity of transporters for L-valine and sucrose (Fig. 2*b*,*c*). Characteristics of [^{14}C] sucrose uptake, however, were quite different whether sorbitol or sucrose was used as an osmoticum.

H^+/Sucrose Symport

The uptake of sucrose into sorb-vesicles was dependent on ΔpH, could be further stimulated by $\Delta\psi$, and was completely abolished by the protonophore

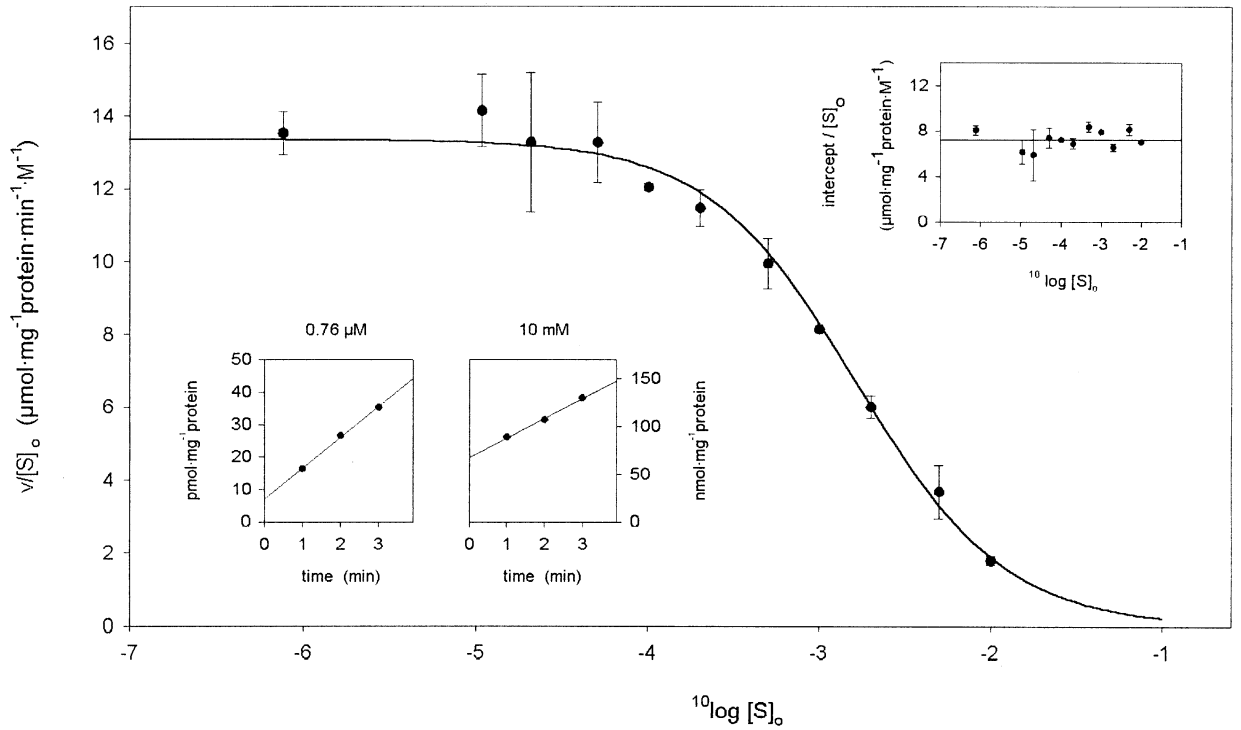

Fig. 6. Concentration dependence of the sucrose influx into 'suc-vesicles'. Uptake was determined after the imposition of Δ pH by diluting the vesicle suspension (in pH7 K-medium) into the incubation medium (pH5 K-medium). Data points represent mean values for two preparations. Lines representing the time-dependent uptake made distinct intercepts with the y-axis (*insets at lower left*). These intercepts were proportional to the external sucrose concentration; the mean value of the proportionality constant amounts to 6.9 ± 0.5 µmol mg^{-1} protein M^{-1} (*inset at upper right*), which corresponds with 5.2 pmol mg^{-1} protein at 0.76 µM. A fit of the Michaelis-Menten equation to the data relating influx and external sucrose yielded the following parameter values: V_{max} = 22.0 ± 0.9 nmol mg^{-1} protein min^{-1}; K_m = 1.65 0.08 mM.

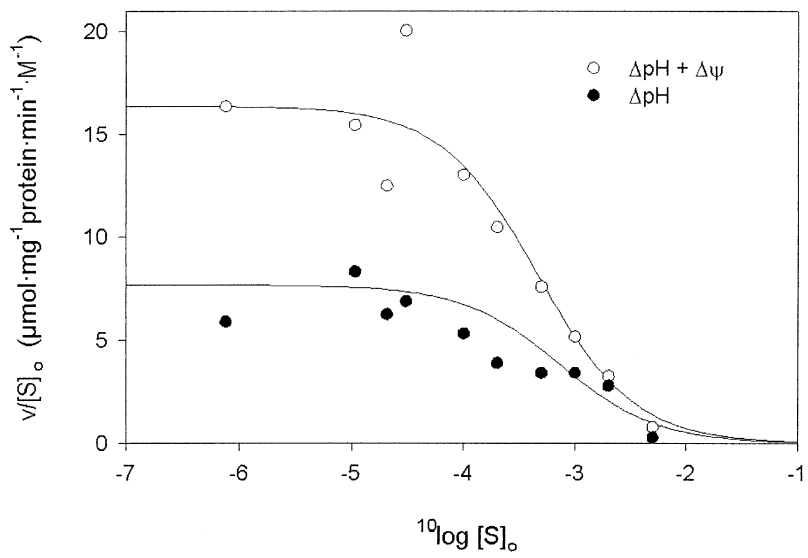

Fig. 7. Concentration dependencies of the sucrose influx into 'sorb-vesicles'. Uptake was determined after imposition of ΔpH + Δψ (○) or ΔpH alone (●). The vesicle suspension in pH7K-medium was diluted into pH5Na-medium (○) or pH5 K-medium (●). Data points represent mean values for two preparations. Kinetic parameters after the imposition of ΔpH: V_{max} = 5.46 ± 0.76 nmol mg^{-1} protein min^{-1}; K_m = 0.69 ± 0.12 mM and after the imposition of ΔpH + Δψ: V_{max} = 7.56 ± 0.33 nmol mg^{-1} protein min^{-1}; K_m = 0.46 ± 0.02 mM.

CCCP. As in previous studies this may be taken as evidence for the electrogenic proton-symport of sucrose (Buckhout, 1989; Bush, 1989; Lemoine & Delrot, 1989; Williams et al., 1990; Marquat, Pétel & Gendraud, 1997). The three- to four-fold stimulation of the initial sucrose influx by Δψ = −68 mV indicates a 1:1 stoichiometry of the proton/sucrose symport (Borstlap & Schuurmans, 2000).

Sucrose transport into plasma membrane vesicles from tobacco leaves showed simple Michaelis-Menten kinetics (Fig. 7), indicating that a single sucrose transporter is involved. We found no indication for

Table 2. Kinetic parameters of sucrose uptake in plasma membrane vesicles

Temp (°C)	Gradients	K_m (mM)	V_{max}	Plant material	References
10–12	Δ pH	1.20	2.25	sugar beet leaves	Bush, 1989
10	Δ pH	0.45	0.153	sugar beet leaves	Buckhout, 1989
23	Δ pH + Δψ	0.3	360	sugar beet leaves	Lemoine & Delrot, 1989
22	Δ pH + Δψ	0.87	45	castor bean cotyledons	Williams et al., 1990
10	Δ pH + Δψ	1.2	1.5	sugar beet leaves	Slone & Buckhout, 1991
23	Δ pH + Δψ	0.5	40	sugar beet leaves	Sakr et al., 1993
10	Δ pH + Δψ	0.51	2.98	sugar beet leaves	Buckhout, 1994
23	Δ pH + Δψ	0.4[a]	22[a]	potato leaves	Lemoine et al., 1996
23	Δ pH + Δψ	0.36[b]	1.0[b]	peach leaves	Marquat et al., 1997
20	Δ pH + Δψ	1.65[c]	22.0[c]	tobacco leaves	this study; Fig. 6
20	Δ pH	0.69	5.46	tobacco leaves	this study; Fig. 7
20	Δ pH + Δψ	0.46	7.56	tobacco leaves	this study; Fig. 7

Unless indicated otherwise, sorbitol was the osmoticum in all media used in the isolation procedure and in the uptake experiments. K_m is expressed in mM and V_{max} in nmol mg^{-1} protein min^{-1}. In vesicles that were isolated using media with sorbitol or mannitol as an osmoticum, the mean K_m of sucrose transport amounts to 0.63 ± 0.10 mM [$n = 11$].

[a] Proton-motive-force-dependent sucrose uptake included a linear component with a proportionality constant of ~20 nmol mg^{-1} protein min^{-1} mM^{-1}.

[b] Media used for the isolation of the vesicles and in uptake experiments contained mannitol as an osmoticum.

[c] Vesicles were isolated using the sucrose-containing media.

sigmoid kinetics, which was reported in one study of sucrose transport into plasma membrane vesicles from sugar beet leaves (Lemoine & Delrot, 1989). Though the possibility of several H^+/sucrose symporters with similar K_ms cannot be excluded, it is clear that the activity of a low-affinity transporter, such as an SUT4-type symporter, could not be detected.

In leaves, all sucrose transporters examined so far localize either to sieve elements or their companion cells. Whether SUTs are present in mesophyll cells is still a matter of debate. It seems reasonable to attribute the H^+/symport activity in plasma membrane vesicles from tobacco leaves to NtSUT1, the orthologue of StSUT1 from potato, which localizes to sieve elements (Bürkle et al., 1998; Kühn et al., 1997). Our vesicle experiments showed that the K_m decreased and V_{max} increased as a function of negative membrane potentials (Table 2). Similar results have been obtained with StSUT1 as well as with AtSUC1 from *Arabidopsis* after expression in *Xenopus* oocytes (Boorer et al., 1996; Zhou et al., 1997).

Table 2 summarizes the results of studies of sucrose transport in plasma membrane vesicles from leaves. Uptake has been measured at various temperatures, pH-gradients, and electrical potentials. All studies revealed only a single saturable system, and although widely divergent V_{max} values were obtained it is noteworthy that the K_ms cluster around a value of 0.6 mM.

Transport by Counter Exchange

Transport of [^{14}C]sucrose into suc-vesicles differed in several respects from that in sorb-vesicles. Most importantly, even in the presence of the protonophore CCCP, the labeled sucrose was accumulated to a level of ~70 pmol mg^{-1} protein (Fig. 4*b*), almost the same as in sorb-vesicles after the imposition of Δ pH + Δψ (Fig. 3*a*,4*a*,5*a*). Furthermore, the imposition of Δψ enhanced the initial influx by only 10% or less, in contrast to sorb-vesicles in which a three- to four-fold enhancement was observed (Fig. 3, 5). These different characteristics of the uptake of the labeled sucrose must be due to the presence of unlabeled sucrose in the vesicles. Apparently the H^+/sucrose transporter facilitates the exchange of the externally supplied [^{14}C]sucrose and of internal, unlabeled sucrose entrapped in the vesicles during their isolation. Interestingly, this uptake was dependent on a relatively high H^+ concentration and barely occurred at pH 7 (Fig. 3*b*,4*b*), which indicates that the symporter has to be in its protonated form.

The kinetic model for StSUT1 is of the LF^- type (Boorer et al., 1996). In this model the proton binds before the substrate molecule, and the empty transporter carries a unit negative charge (Fig. 8a). Exchange can be envisaged to occur by the protonated form of the symporter, oscillating back and forth between inwardly and outwardly facing conformations, binding and releasing sucrose on alternate sides of the membrane (Fig. 8*b*).

Our results provide the first evidence for counter exchange by a plant H^+/sucrose symporter. In fact, it could have been predicted from the quantitative kinetic model for StSUT1; in which the rate constants for the transmembrane movements of the loaded carrier were both determined at 50 s^{-1} (Boorer et al., 1996). The model also predicts that at the inside of the membrane sucrose dissociates much faster from

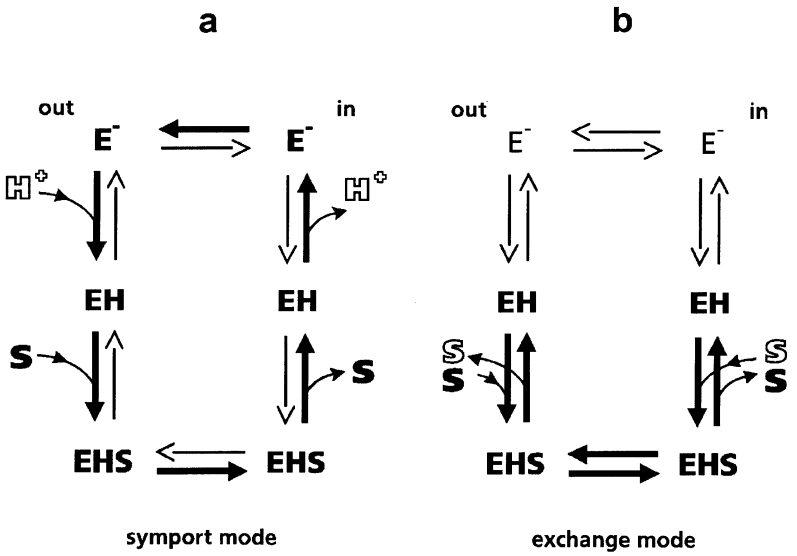

Fig. 8. Kinetic model for the H^+/sucrose transporter operating in the symport mode and in the exchange mode. Bold arrows indicate the presumed route of sucrose transport. The model is of the LF^--type (Sanders et al., 1984), in which the substrate binds last and dissociates first, and the loaded transporter is electrically neutral. Boorer et al. (1996) adopted the model to describe the kinetics of StSUT1, but in addition included a transmembrane step for the protonated transporter and a route for the transport of sucrose without protons, which are not shown here. They also identified the reorientation of the empty, negatively charged transporter, and the binding and release of the proton as the $\Delta\psi$-dependent steps in the symport mode.

the transporter than does the proton (rate constants of 1×10^3 s^{-1} and 4.3 s^{-1}, respectively). These characteristics, together with a high sucrose concentration in the vesicles will favor the exchange of sucrose molecules as envisaged in Fig. 8*b*.

In principle any symporter can exchange substrate molecules across the membrane. Actually this is what happens at equilibrium when the outwardly directed substrate gradient is balanced by the inwardly directed electrochemical gradient of the driver ion and, consequently, the influx and efflux of substrate and driver ion(s) are equal. Hopfer & Groseclose (1980) measured substrate influx under equilibrium exchange conditions for the Na^+ -dependent transport of glucose in rabbit brush border membranes. Heteroexchange, the exchange of different substrates, has been reported for Na^+ -dependent anionic amino-acid transporters from animal cells (Zerangue & Kavanaugh, 1996; Millar et al., 1997). Theoretically, the exchange of substrate molecules *without* driver ions is only possible for symporters that conform to the LF model (Fig. 8*b*). This type of exchange has been extensively described for another H^+/disaccharide symporter, the lactose carrier from *Escherichia coli* (for a review, *see* Kaback, Sahin-Tóth & Weinglass, 2001).

There are several indications that the transport of labeled sucrose into the vesicles started by the exchange mechanism and was gradually replaced by the symport route. First, CCCP had no effect on the initial influx, but it did prevent further uptake when added after 20 min of incubation (Fig. 4*b*). Secondly, the imposition of $\Delta\psi$ in addition to ΔpH did not stimulate the initial influx though it resulted in slightly higher levels of accumulation (Fig. 3*b*,5*b*). Thirdly, in the absence of CCCP and after 1 h of incubation the uptake of labeled sucrose was about 50 pmol mg^{-1} protein higher than in the presence of the protonophore. This difference is comparable with the accumulation of labeled sucrose in sorb-vesicles after the imposition of ΔpH + $\Delta\psi$ and 1 h of incubation.

It may be emphasized that the activity of an H^+ -symporter operating in the exchange mode does not affect the maximum accumulation of its substrate at the prevailing proton motive force. It should not be confused with an uncoupled route for transport of the substrate, as has been proposed for StSUT1 (Boorer et al., 1996), which is in effect an internal leakage pathway and lowers the maximum accumulation that can be accomplished by the symporter. In the exchange mode, the activity of the transporter is comparable to that of a transporter of the exchange-only type, facilitating the 1:1 exchange of substrate molecules across the membrane (Stein, 1986).

No Linear Component

To determine the uptake in the vesicles the external medium is removed by washing of the vesicle samples collected on the filters. This washing procedure is always a compromise. On the one hand, the external label should be removed as much as possible, on the other hand, exit of solute from the vesicles during the washing should be kept at a minimum. In our experiments the external label was probably not completely removed, as evidenced by the 'time-independent uptake' of sucrose (Fig. 6, *insets at lower left*). It corresponds with as little as ~0.05% of the radioactivity in the 0.2-ml samples of incubation mixture from which vesicles were collected in the uptake assay. Similar amounts (0.03 to 0.06%) of residual radioactivity have been found in studies with vesicles from seed coats and cotyledons of developing

pea seeds (De Jong & Borstlap, 2000a,b). It is not impossible that the linear component of sucrose uptake observed in previous vesicle studies (Buckhout, 1989; Lemoine & Delrot, 1989; Williams et al., 1990) in fact resulted from residual extravesicular label.

Alternatively, the 'time-independent uptake', amounting to 5.2 pmol mg^{-1} protein at an external substrate concentration of 0.76 μM (*see* legend of Fig. 6), may be interpreted as a very fast transport into the vesicles by a passive mechanism. This would then result in a transport component that depends linearly on the external substrate concentration. The presence of such a fast transport route in the vesicle membrane, however, is incompatible with the accumulation of the labeled sucrose up to levels of 60 to 90 pmol mg^{-1} protein that were recorded after the imposition of $\Delta pH + \Delta\psi$ (Figs. 3*a*,4*a*,5*a*).

The linear component in the concentration dependency of sucrose uptake by leaf tissues contributes some 20% to the total sucrose influx, as is apparent from a comparison of its proportionality constant k and the ratio V_{max}/K_m of the saturable component (Table 1). Clearly, this linear component did not show up in our experiments with plasma membrane vesicles, neither when the sucrose transporter was acting in the symport mode, nor when it was acting in the exchange mode (Figs. 6, 7). We conclude, therefore, that the transport route represented by the linear component of sucrose uptake by leaf tissues is not provided by the sucrose transporter itself but by a different transporter. The suggestion of Boorer et al. (1996) that this linear component might represent uncoupled sucrose transport by the symporter is not supported by our data. Either the transporter responsible for the linear component in sucrose uptake by leaf tissue was inactive in our membrane preparations, or its activity was undetectable because, due to a very low abundance, it was present in only a very minor fraction of the vesicle population.

H^+/Sucrose Symporters as Effluxers

Could H^+/sucrose symporters in the sieve elements of sink organs play a role in apoplasmic phloem unloading? This depends on two factors. First, the rate of efflux by the symporter should be sufficient to meet the demand of the sucrose-utilizing cells in the sink. Sucrose symporters may well fulfill this requirement since there is a long-standing notion that sugar transporters are not subjected to transinhibition, but rather exhibit a progressively increased efflux as the substrate is accumulated (Sauer, Komor & Tanner, 1983; Sanders, 1990). Our results show substantial transmembrane sucrose fluxes by counter exchange, but data of sucrose efflux via a plant H^+/sucrose symporter at or near equilibrium are still not available.

Secondly, the concentration of sucrose in the sink apoplasm could be a determining factor for the utilization of sucrose by cells of sink organs. Though uncoupled sucrose transport by the symporter does not necessarily result in higher efflux rates, it is likely to result in higher sucrose concentrations in the sink apoplasm. However, such uncoupled transport by SUTs is questionable. Boorer et al. (1996) simultaneously measured the influx of [^{14}C]sucrose and inward movement of electric charge into oocytes in which *StSUT1* was expressed at different levels. Plotting these measurements against each other revealed a linear relationship with an intercept that indicated the influx of sucrose without protons (Fig. 6 *C* in Boorer et al., 1996). This interpretation implies that the proportion of uncoupled and proton-coupled transport of sucrose would depend on the expression level of *StSUT1*, which seems quite unlikely.

When the transport of sucrose and protons by the symporter is tightly coupled, sucrose concentrations in the sink apoplasm will be rather low. If, for example, the sucrose concentration in the sieve elements amounts to 300 mM, $\Delta pH = 2$ and $\Delta\psi = -116$ mV, then the apoplasmic concentration at equilibrium is predicted at 30 μM. But there is no reason to assume that this concentration is too low for the proper utilization of sucrose by parenchyma cells in the sink organ, not in the least when these cells have H^+/sucrose symporters in their plasma membranes.

Although counterintuitive at first sight, it seems quite well possible that H^+-symporters sometimes function as effluxers. There is no compelling reason why H^+-symporters in the plasma membranes of sieve elements in sinks could not be used for apoplasmic phloem unloading. Most probably, the presence of an H^+/sucrose transporter will enhance the permeability of the sieve element membrane for sucrose enormously, and this may be much more important than the low sucrose concentration in the apoplasm. Circumstantial evidence for the involvement of StSUT1 in phloem unloading in developing potato tubers was recently presented (Kühn et al., 2003), and a few SUTs have already been localized to parenchyma cells of sink organs (Shakya & Sturm, 1998; Flemetakis et al., 2003). Two symporters in series, one for efflux from the sieve element and one for accumulation of sucrose by sink parenchyma cells, could be one possible way by which the acquisition of sucrose by sink tissues is achieved.

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